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Exhibit "D" attached to Declaration of Vishwanath R. Iyer, Ph.D.

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co mosaic viral RNA was obtained by phenol and chloroform extractions of the virus and precipitated from ethanol. CA-NC assembly reactions in the presence of noncognate RNAs were identical to those given in (9). In the absence of RNA, CA-NC cones formed under the following conditions: 300 μ M CA-NC, 1 M NaCl, and 50 mM tris-HCl (pH 8.0) at 37°C for 60 min. In the absence of exogenous RNA, neither cones nor cylinders formed at concentrations of 0.5 M NaCl or below. Absorption spectra demonstrated that our CA-NC preparations were not contaminated with *Escherichia coli* RNA (estimated lower detection limit was \sim 1 base/protein molecule). To control for even lower levels of RNA contamination, we preincubated the CA-NC protein with 0.5 mg/ml ribonuclease A (Type 1-A5, 54 Kunitz U/mg, Sigma) for 1 hour at 4°C, which then formed cones normally.

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17. Assembly mixtures were deposited on holey carbon grids, blotted briefly with filter paper, plunged into liquid ethane, and transferred to liquid nitrogen. Frozen grids were transferred to a Philips 420 TEM equipped with a Gatan cold stage system, and images of particles in vitreous ice were recorded under low dose conditions at 36,000 \times magnification and \sim 1.6- μ m defocus.

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24. Single-letter abbreviations for the amino acid residues are as follows: A: Ala; C: Cys; D: Asp; E: Glu; F: Phe; G: Gly; H: His; I: Ile; K: Lys; L: Leu; M: Met; N: Asn; P: Pro; Q: Gln; R: Arg; S: Ser; T: Thr; V: Val; W: Trp; and Y: Tyr.

25. We thank C. Hill for very helpful discussions on the relationship between viral cores and fullerene cones, D. Hobbs for refining the ChemDraw3D images of cones, G. Stubbs for a gift of tobacco mosaic virus, J. McCutcheon for the plasmid used to prepare ribosomal RNA, and K. Albertine and N. Chandler of the University of Utah Shared Electron Microscopy facility for their support and encouragement. Supported by grants from NIH and from the Huntsman Cancer Institute (to W.I.S.).

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The Transcriptional Program in the Response of Human Fibroblasts to Serum

Vishwanath R. Iyer, Michael B. Eisen, Douglas T. Ross, Greg Schuler, Troy Moore, Jeffrey C. F. Lee, Jeffrey M. Trent, Louis M. Staudt, James Hudson Jr., Mark S. Boguski, Deval Lashkari, Dari Shalon, David Botstein, Patrick O. Brown*

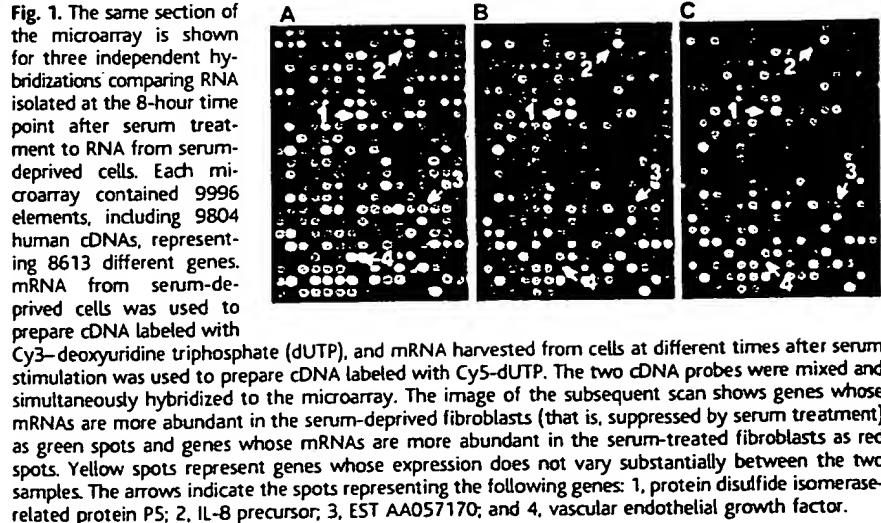
The temporal program of gene expression during a model physiological response of human cells, the response of fibroblasts to serum, was explored with a complementary DNA microarray representing about 8600 different human genes. Genes could be clustered into groups on the basis of their temporal patterns of expression in this program. Many features of the transcriptional program appeared to be related to the physiology of wound repair, suggesting that fibroblasts play a larger and richer role in this complex multicellular response than had previously been appreciated.

The response of mammalian fibroblasts to serum has been used as a model for studying growth control and cell cycle progression (1). Normal human fibroblasts require growth factors for proliferation in culture; these growth factors are usually provided by fetal

bovine serum (FBS). In the absence of growth factors, fibroblasts enter a nondividing state, termed G_0 , characterized by low

metabolic activity. Addition of FBS or purified growth factors induces proliferation of the fibroblasts; the changes in gene expression that accompany this proliferative response have been the subject of many studies, and the responses of dozens of genes to serum have been characterized.

We took a fresh look at the response of human fibroblasts to serum, using cDNA microarrays representing about 8600 distinct human genes to observe the temporal program of transcription that underlies this response. Primary cultured fibroblasts from human neonatal foreskin were induced to enter a quiescent state by serum deprivation for 48 hours and then stimulated by addition of medium containing 10% FBS (2). DNA microarray hybridization was used to measure the temporal changes in mRNA levels of 8613 human genes (3) at 12 times, ranging from 15 min to 24 hours after serum stimulation. The cDNA made from purified mRNA from each sample was labeled with the fluorescent dye Cy5 and mixed with a common reference probe consisting of cDNA made from purified mRNA from the quiescent



V. R. Iyer and D. T. Ross, Department of Biochemistry, Stanford University School of Medicine, Stanford CA 94305, USA. M. B. Eisen and D. Botstein, Department of Genetics, Stanford University School of Medicine, Stanford CA 94305, USA. G. Schuler and M. S. Boguski, National Center for Biotechnology Information, Bethesda MD 20894, USA. T. Moore and J. Hudson Jr., Research Genetics, Huntsville, AL 35801, USA. J. C. F. Lee, D. Lashkari, D. Shalon, Incyte Pharmaceuticals, Fremont, CA 94555, USA. J. M. Trent, Laboratory of Cancer Genetics, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA. L. M. Staudt, Metabolism Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, MD 20892, USA. P. O. Brown, Department of Biochemistry and Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford CA 94305, USA.

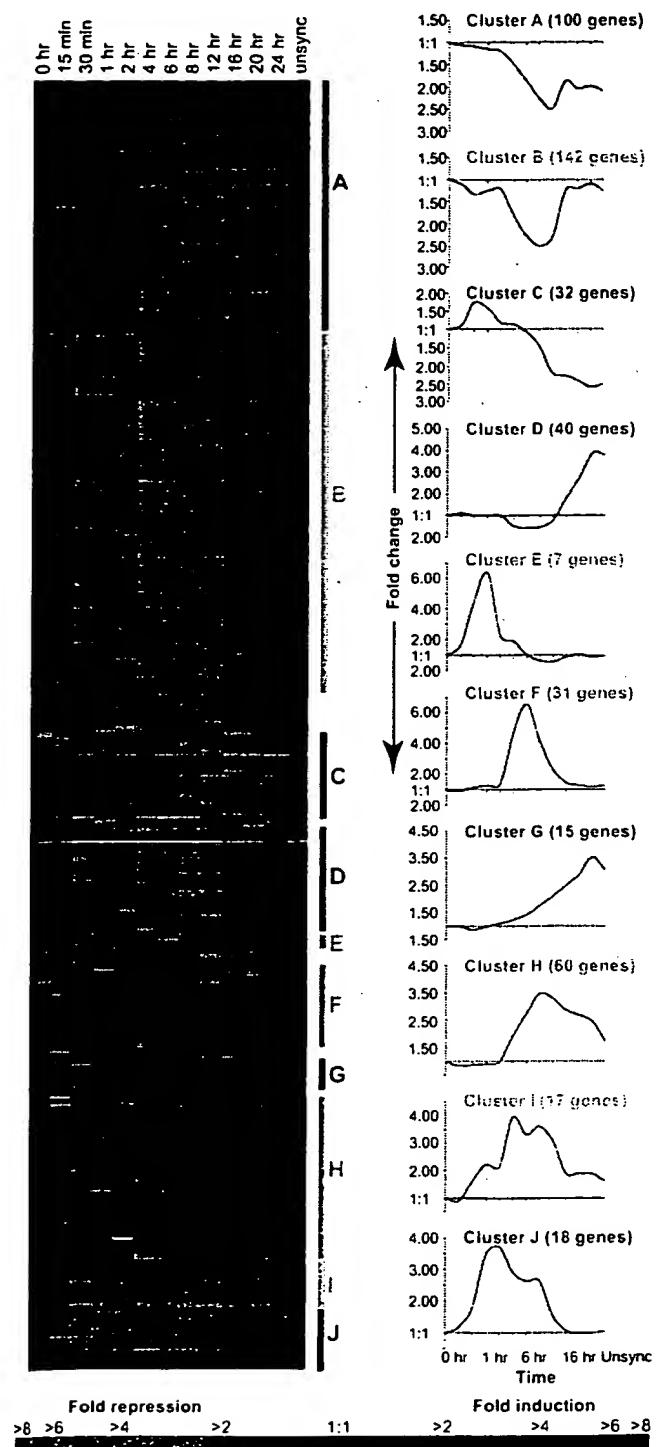
*To whom correspondence should be addressed. E-mail: pbrown@cmgm.stanford.edu

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culture (time zero) labeled with a second fluorescent dye, Cy3 (4). The color images of the hybridization results (Fig. 1) were made by representing the Cy3 fluorescent image as green and the Cy5 fluorescent image as red and merging the two color images.

Diverse temporal profiles of gene expression could be seen among the 8613 genes sur-

Fig. 2. Cluster image showing the different classes of gene expression profiles. Five hundred seventeen genes whose mRNA levels changed in response to serum stimulation were selected (7). This subset of genes was clustered hierarchically into groups on the basis of the similarity of their expression profiles by the procedure of Eisen et al. (6). The expression pattern of each gene in this set is displayed here as a horizontal strip. For each gene, the ratio of mRNA levels in fibroblasts at the indicated time after serum stimulation ("unsync" denotes exponentially growing cells) to its level in the serum-deprived (time zero) fibroblasts is represented by a color, according to the color scale at the bottom. The graphs show the average expression profiles for the genes in the corresponding "cluster" (indicated by the letters A to J and color coding). In every case examined, when a gene was represented by more than one array element, the multiple representations in this set were seen to have identical or very similar expression profiles, and the profiles corresponding to these independent measurements clustered either adjacent or very close to each other, pointing to the robustness of the clustering algorithm in grouping genes with very similar patterns of expression.



veyed in this experiment (Fig. 2); many of these genes (about half) were unnamed expressed sequence tags (ESTs) (5). Although diverse patterns of expression were observed, the orderly choreography of the expression program became apparent when the results were analyzed by a clustering and display method developed in our laboratory for analyzing genome-wide

gene expression data (6). An example of such an analysis, here applied to a subset of 517 genes whose expression changed substantially in response to serum (7), is shown in Fig. 2. The entire detailed data set underlying Fig. 2 is available as a tab-delimited table (in cluster order) at the *Science* Web site (www.sciencemag.org/feature/data/984559.shl). In addition, the entire, larger data set for the complete set of genes analyzed in this experiment can be found at a Web site maintained by our laboratory (genome-www.stanford.edu/serum) (8).

One measure of the reliability of the changes we observed is inherent in the expression profiles of the genes. For most genes whose expression levels changed, we could see a gradual change over a few time points, which thus effectively provided independent measurements for almost all of the observations. An additional check was provided by the inclusion of duplicate and, in a few cases, multiple array elements representing the same gene for about 5% of the genes included in this microarray. In addition, three independent hybridizations to different microarrays with mRNA samples from cells harvested 8 hours after serum addition showed good correlation (Fig. 1). As an independent test, we measured the expression levels of several genes using the TaqMan 5' nuclease fluorogenic quantitative polymerase chain reaction (PCR) assay (9). The expression profiles of the genes, as measured by these two independent methods, were very similar (Fig. 3) (10).

The transcriptional response of fibroblasts to serum was extremely rapid. The immediate response to serum stimulation was dominated by genes that encode transcription factors and other proteins involved in signal transduction. The mRNAs for several genes [including c-FOS, JUN B, and mitogen-activated protein (MAP) kinase phosphatase-1 (MKP1)] were detectably induced within 15 min after serum stimulation (Fig. 4, A and B). Fifteen of the genes that were observed to be induced by serum encode known or suspected regulators of transcription (Fig. 4B). All but one were immediate-early genes—their induction was not inhibited by cycloheximide (11). This class of genes could be distinguished into those whose induction was transient (Fig. 2, cluster E) and those whose mRNA levels remained induced for much longer (Fig. 2, clusters I and J). Some features of the immediate response appeared to be directed at adaptation to the initiating signals. We observed a marked induction of mRNA encoding MKP1, a dual-specificity phosphatase that modulates the activity of the ERK1 and ERK2 MAP kinases (12). The coincidence of the peak of expression of genes in cluster E (Fig. 2) with that of MKP1 (Fig. 4A) suggests the possibility

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that continued activity of the MAP kinase pathway is required to maintain induction of these genes but not of those with sustained expression (clusters I and J). The gene encoding a second member of the dual-specificity MAP kinase phosphatase family, known as dual-specificity protein phosphatase 6/pyst2, was induced later, at about 4 hours after serum stimulation. Genes encoding diverse other proteins with roles in signal transduction, ranging from cell-surface receptors [for example, the sphingosine 1-phosphate receptor (EDG-1), the vascular endothelial growth factor receptor, and the type II BMP receptor] to regulators of G-protein signaling (for example, NET1/p115 rho GEF) to DNA-binding transcription factors, were induced by serum (Fig. 4A).

The reprogramming of the regulatory circuits in response to serum involved not only induction of transcription factors but also reduced expression of many transcriptional regulators—some of which may play roles in maintaining the cells in G_0 or in priming them to react to wounding (Fig. 4C). Perhaps as a consequence of the historical focus on genes induced by serum stimulation of fibroblasts, the set of transcription factors whose expression diminished upon serum stimulation has been less well characterized.

Genes known or likely to be involved in controlling and mediating the proliferative response showed distinctive patterns of regulation. Several genes whose products inhibit progression of the cell-division cycle, such as p27 Kip1, p57 Kip2, and p18, were expressed in the quiescent fibroblasts and down-regulated before the onset of cell division. The nadir in the mRNA levels for these genes occurred between 6 and 12 hours after serum stimulation (Fig. 5A), coincident with the passage of the fibroblasts through G_1 . The levels of the transcript encoding the WEE1-like protein kinase, which is believed to inhibit mitosis by phosphorylation of Cdc2, diminished between 4 and 8 to 12 hours after serum addition (Fig. 5A), well

before the onset of M phase at around 16 hours, raising the possibility of an additional role for Wee1 in an earlier stage of the cell cycle or in regulating the G_0 to G_1 transition. Several genes induced in the first few hours after serum stimulation, such as the helix-loop-helix proteins ID2 and ID3 and EST AA016305, a gene with homology to G_1 -S cyclins, are candidates for roles in promoting the exit from G_0 .

Genes involved in mediating progression through the cell cycle were characterized by a distinctive pattern of expression (Fig. 2, cluster D), reflecting the coincidence of their expression with the reentry of the stimulated fibroblasts into the cell-division cycle. The stimulated fibroblasts replicated their DNA about 16 hours after serum treatment. This timing was reflected by the induction of mRNA encoding both subunits of ribonucleotide reductase and PCNA, the processivity factor for DNA polymerase epsilon and delta. Cyclin A, Cyclin B1, Cdc2, and CDC28 kinase, regulators of passage through the S phase and the transition from G_2 to M phase, were induced at about 16 to 20 hours after serum addition. The kinase in the Cyclin B1-CDK pair needs to be activated by phosphorylation. The gene encoding Cyclin-dependent kinase 7 (CDK7; a homolog of *Xenopus* MO15 cdk-activating kinase) was induced in parallel with the Cdc2 and Cdc28 kinases (Fig. 5A), suggesting a potential role for CDK7 in mediating M phase. DNA topoisomerase II α , required for chromosome segregation at mitosis; Mad2, a component of the spindle checkpoint that prevents completion of mitosis (anaphase) if chromosomes are not attached to the spindle; and the kinetochore protein CENP-F all showed a similar expression profile.

In the hours after the serum stimulus, one of the most striking features of the unfolding transcriptional program was the appearance of numerous genes with known roles in processes relevant to the physiology of wound healing.

These included both genes involved in the direct role played by fibroblasts in remodeling of the clot and the extracellular matrix and, more notably, genes encoding proteins involved in intercellular signaling (Fig. 5). Genes induced in this program encode products that can (i) participate in the dynamic process of clotting, clot dissolution, and remodeling and perhaps contribute to hemostasis by promoting local vasoconstriction (for example, endothelin-1); (ii) promote chemotaxis and activation of neutrophils (for example, COX2) and recruitment and extravasation of monocytes and macrophages (for example, MCP1); (iii) promote chemotaxis and activation of T lymphocytes [for example, interleukin-8 (IL-8)] and B lymphocytes (for example, ICAM-1), thus providing both innate and antigen-specific defenses against wound infection and recruiting the phagocytic cells that will be required to clear out the debris during remodeling of the wound; (iv) promote angiogenesis and neovascularization (for example, VEGF) through newly forming tissue; (v) promote migration and proliferation of fibroblasts (for example, CTGF) and their differentiation into myofibroblasts (for example, Vimentin); and (vi) promote migration and proliferation of keratinocytes, leading to reepithelialization of the wound (for example, FGF7), and promote proliferation of melanocytes, perhaps contributing to wound hyperpigmentation (for example, FGF2).

Coordinated regulation of groups of genes whose products act at different steps in a common process was a recurring theme. For example, Furin, a prohormone-processing protease required for one of the processing steps in the generation of active endothelin, was induced in parallel with induction of the gene encoding the precursor of endothelin-1 (Fig. 5E) (13). Conversely, expression of CALLA/CD10, a membrane metalloprotease that degrades endothelin-1 and other peptide mediators of acute inflammation, was re-

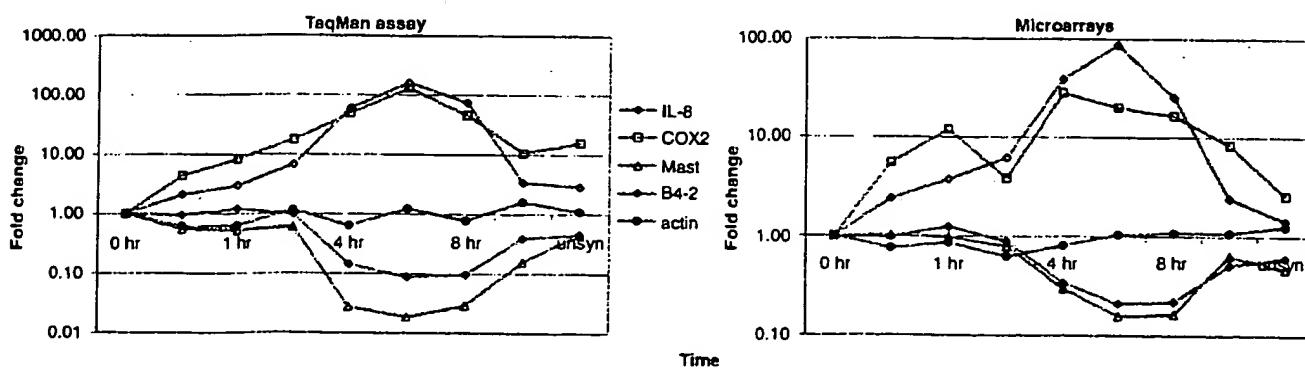


Fig. 3. Independent verification of microarray quantitation. Relative mRNA levels of the indicated genes (Mast, mast/stem cell growth factor receptor) were measured with the TaqMan 5' nuclease fluorogenic quantitative PCR assay (9) (left) in the same samples that were used to prepare probes for microarray hybridizations (right). Data from the TaqMan analysis were

normalized to mRNA concentrations and plotted relative to the level at time zero, so that the results could be compared with those from the microarray hybridizations. In general, quantitation with the two methods gave very similar results (10).

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duced. A second example is provided by a set of five genes involved in the biosynthesis of cholesterol (Fig. 5I). The mRNAs encoding each of these enzymes showed sharply diminished expression beginning 4 to 6 hours after serum stimulation of fibroblasts. A likely explanation for the coordinated down-regulation of the cholesterol biosynthetic pathway is that serum provides cholesterol to fibroblasts through low-density lipoproteins, whereas in the absence of the cholesterol provided by serum, endogenous cholesterol biosynthesis in fibroblasts is required.

Many of the previously studied genes that we observed to be regulated in this program have no recognized role in any aspect of wound healing or fibroblast proliferation. Their identification in this study may therefore point to previously unknown aspects of these processes. A few selected genes in this group are shown in Fig. 5H. The stanniocalcin gene, for example (Fig. 5H), encodes a secreted protein without a clearly identified function in human cells (14, 15). Its induction in serum-stimulated fibro-

blasts suggests the possibility that it may play a role in the wound-healing process, perhaps serving as a signal in mediating inflammation or angiogenesis.

One of the most important results of this exploration was the discovery of over 200 previously unknown genes whose expression was regulated in specific temporal patterns during the response of fibroblasts to serum. For example, 13 of the 40 genes in cluster D (Fig. 2) have descriptive names that reflect their putative function. Nine of these 13 genes (69%) encode proteins that play roles in cell cycle progression, particularly in DNA replication and the G₂-M transition. This enrichment for cell cycle-related genes suggests that some of the

unnamed genes in this cluster—for example, EST W79311 and EST R13146, neither of which have sequence similarity to previously characterized genes—may represent previously unknown genes involved in this part of the cell cycle. Similarly, a remarkable fraction of genes that were grouped into cluster F on the basis of their expression profiles encoded proteins involved in intercellular signaling (Fig. 2), suggesting that a similar role should be considered for the many unnamed genes in this cluster. A disproportionately large fraction of the genes whose transcription diminished upon serum stimulation were unnamed ESTs.

Our intention was to use this experiment as a model to study the control of the transition

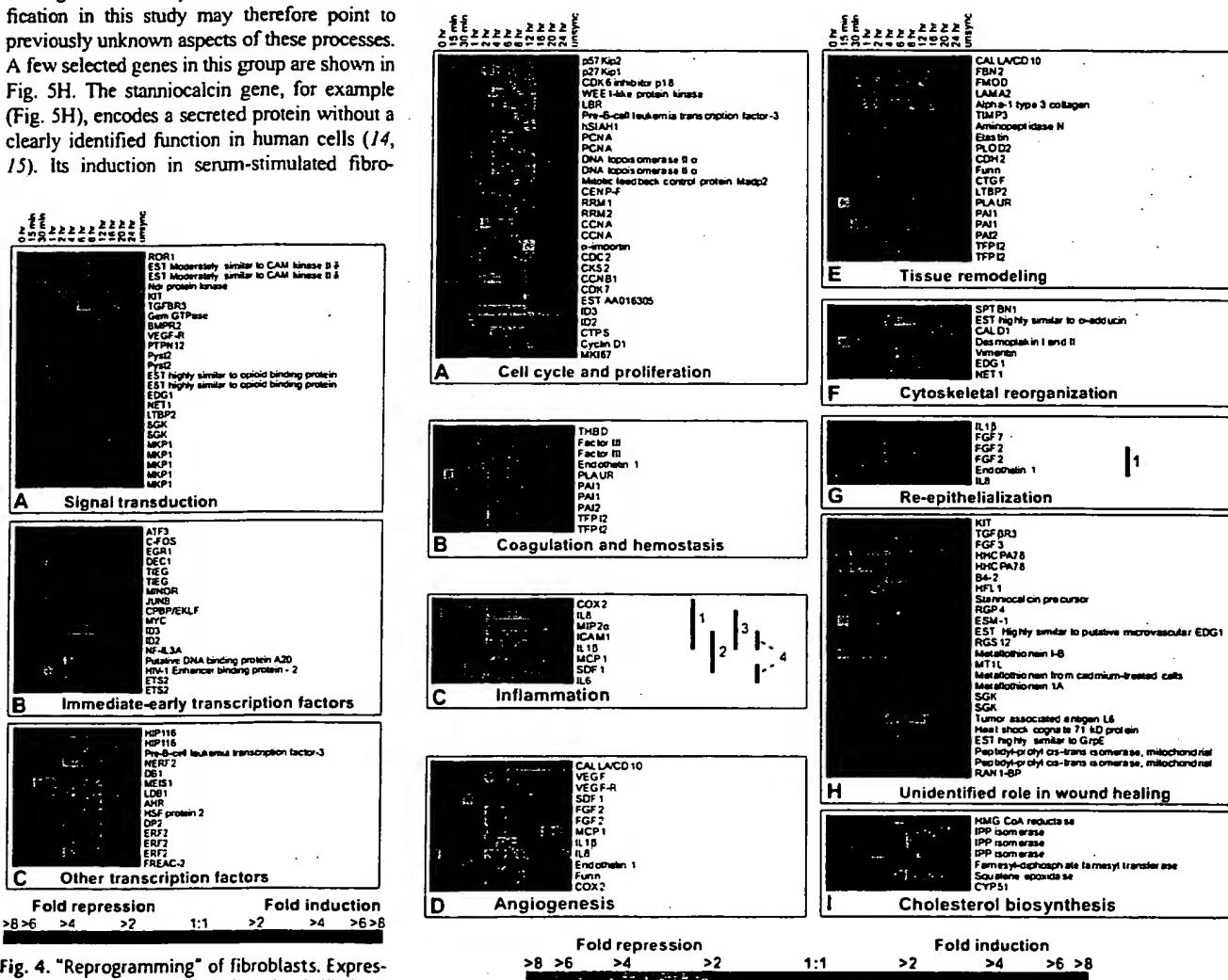


Fig. 4. "Reprogramming" of fibroblasts. Expression profiles of genes whose function is likely to play a role in the reprogramming phase of the response are shown with the same representation as in Fig. 2. In the cases in which a gene was represented by more than one element in the microarray, all measurements are shown. The genes were grouped into categories on the basis of our knowledge of their most likely role. Some genes with pleiotropic roles were included in more than one category.

Fig. 5. The transcriptional response to serum suggests a multifaceted role for fibroblasts in the physiology of wound healing. The features of the transcriptional program of fibroblasts in response to serum stimulation that appear to be related to various aspects of the wound-healing process and fibroblast proliferation are shown with the same convention for representing changes in transcript levels as was used in Figs. 2 and 4. (A) Cell cycle and proliferation, (B) coagulation and hemostasis, (C) inflammation, (D) angiogenesis, (E) tissue remodeling, (F) cytoskeletal reorganization, (G) reepithelialization, (H) unidentified role in wound healing, and (I) cholesterol biosynthesis. The numbers in (C) and (G) refer to genes whose products serve as signals to neutrophils (C1), monocytes and macrophages (C2), T lymphocytes (C3), B lymphocytes (C4), and melanocytes (G1).

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from G_0 to a proliferating state. However, one of the defining characteristics of genome-scale expression profiling experiments is that the examination of so many diverse genes opens a window on all the processes that actually occur and not merely the single process one intended to observe. Serum, the soluble fraction of clotted blood, is normally encountered by cells in vivo in the context of a wound. Indeed, the expression program that we observed in response to serum suggests that fibroblasts are programmed to interpret the abrupt exposure to serum not as a general mitogenic stimulus but as a specific physiological signal, signifying a wound. The proliferative response that we originally intended to study appeared to be part of a larger physiological response of fibroblasts to a wound. Other features of the transcriptional response to serum suggest that the fibroblast is an active participant in a conversation among the diverse cells that work together in wound repair, interpreting, amplifying, modifying, and broadcasting signals controlling inflammation, angiogenesis, and epithelial regrowth during the response to an injury.

We recognize that these *in vitro* results almost certainly represent a distorted and incomplete rendering of the normal physiological response of a fibroblast to a wound. Moreover, only the responses elicited directly by exposure of fibroblasts to serum were examined. The subsequent signals from other cellular participants in the normal wound-healing process would certainly provoke further evolution of the transcriptional program in fibroblasts at the site of a wound, which this experiment cannot reveal. Nevertheless, we believe that the picture that emerged strongly suggests a much larger and richer role for the fibroblast in the orchestration of this important physiological process than had previously been suspected.

References and Notes

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2. A normal human diploid fibroblast cell line derived from foreskin (ATCC CRL 2091) in passage 8 was used in these experiments. The protocol followed for growth arrest and stimulation was essentially that of (16) and (17). Cells were grown to about 60% confluence in 15-cm petri dishes in Dulbecco's minimum essential medium containing glucose (1 g/liter), the antibiotics penicillin and streptomycin, and 10% (by vol) FBS (Hyclone) that had been previously heat inactivated at 56°C for 30 min. The cells were then washed three times with the same medium lacking FBS, and low-serum medium (0.1% FBS) was added to the plates. After a 48-hour incubation, the medium was replaced with fresh medium containing 10% FBS. mRNA was isolated from several plates of cells harvested before serum stimulation; this mRNA served as the serum-starved or time-zero reference sample. Cells were harvested from batches of plates at 11 subsequent intervals (15 min, 30 min, 1, 2, 4, 6, 8, 12, 16, 20, and 24 hours) after the addition of serum. mRNA was also isolated from exponentially growing fibroblasts (not subjected to serum starvation). mRNA was isolated with the FastTrack mRNA isolation kit (Invitrogen), which involves lysis of the cells on the plate. The growth medium was removed, and the cells were quickly washed with phosphate-buffered saline at room temperature. The lysis buffer was added to the plate, transferred to tubes, and frozen in liquid nitrogen. Subsequent steps were performed according to the kit manufacturer's protocols.
3. The National Center for Biotechnology Information maintains the UniGene database as a resource for partitioning human sequences contained in GenBank into clusters representing distinct transcripts or genes (18, 19). At the time this work began this database contained about 40,000 such clusters. We selected a subset of 10,000 of these UniGene clusters for inclusion on gene expression microarrays. UniGene clusters were included only if they contained at least one clone from the L.M.A.G.E. human cDNA collection (20), so that a physical clone could easily be obtained (all L.M.A.G.E. clones are available commercially from a number of vendors). We attempted to include as complete as possible a set of the "named" human genes (about 4000) and genes that appeared to be closely related to named genes in other organisms (about an additional 2000). The remaining 4000 clones were chosen from among the "anonymous" UniGene clusters on the basis of inclusion on the human transcript map (www.ncbi.nlm.nih.gov/SCIENCE96/) and the lack of apparent homology to any other genes in the selected set. A physical clone representing each of the selected genes was obtained from Research Genetics. This "10K set" is included in a more recent "15K set" described at www.ncbi.nlm.nih.gov/DIR/LCG/15K/HTML/p15Ktop.html. Of these clones, 472 are absent from the current edition of UniGene and were presumed to be distinct genes. The remainders represent 8141 distinct clusters, or human genes, in UniGene. These clones, thus presumed to represent 8613 different genes, were used to print microarrays according to methods described previously (21, 22).
4. One microgram of mRNA was used for making fluorescently labeled cDNA probes for hybridizing to the microarrays, with the protocol described previously (23). mRNA from the large batch of serum-starved cells was used to make cDNA labeled with Cy3. The Cy3-labeled cDNA from this batch of serum-starved cells served as the common reference probe in all hybridizations. mRNA samples from cells harvested immediately before serum stimulation, at intervals after serum stimulation, and from exponentially growing cells were used to make cDNA labeled with Cy5. Ten micrograms of yeast tRNA, 10 µg of polydeoxyadenylic acid, and 20 µg of human Cot1 DNA (Clontech-BRL) were added to the mixture of labeled probes in a solution containing 3× standard saline citrate (SSC) and 0.3% SDS and allowed to prehybridize at room temperature for 30 min before the probe was added to the surface of the microarray. Hybridizations, washes, and fluorescent scans were performed as described previously (23, 24). All measurements, totaling more than 180,000 differential expression measurements, were stored in a computer database for analysis and interpretation.
5. The nominal identities of a number of cDNAs (currently about 3750) on the microarray were verified by sequencing. The clones that were sequenced included many of the genes whose expression changed substantially upon serum stimulation, as well as a large number of genes whose expression did not change substantially in the course of this experiment. About 85% of the clones on the current version of this microarray that were checked by resequencing were correctly identified. In all the figures, gene names or EST numbers are given only for those genes on the microarrays whose identities were reconfirmed by resequencing. In the cases where a human gene has more than one name in the literature, we have tried to use the name that is most evocative of its presumed role in this context. The remainder of the clones have been assigned a temporary identification number (format: SID# #####) and a putative identity pending sequence verification. The correct identities of these genes will be posted at our Web site (genome-www.stanford.edu/serum) as they are confirmed by resequencing.
6. M. B. Eisen, P. T. Spellman, P. O. Brown, D. Botstein, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14863 (1998).
7. Genes were selected for this analysis if either (i) their expression level deviated from that in quiescent fibroblasts by at least a factor of 2.20 in at least two of the samples from serum-stimulated cells or (ii) the standard deviation for the set of 13 values of \log_2 (expression ratio) measured for the gene in this time course exceeded 0.7. In addition, observations in which the pixel-by-pixel correlation coefficients for the Cy3 and Cy5 fluorescence signals measured in a given array element were less than 0.6 were excluded. This selection criteria yielded a computationally manageable number of genes while minimizing the number of genes that were included because of noise in the data.
8. A more complete analysis and interpretation of the results of this experiment, as well as a searchable database, can be found at genome-www.stanford.edu/serum
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10. The apparent dip in the profile of COX2 at the 2-hour time point in the microarray data appears to result from a localized area of low intensity on the corresponding array scan resulting in an underestimation of the expression ratio. The expression ratios measured for mast/stem cell growth factor receptor are somewhat lower in the microarray data. This discrepancy is probably a consequence of the conservative background subtraction method used for quantitating the signal intensities on the array scans (23). The sequences of the PCR primer pairs (5' to 3') that were used are as follows: COX2, CCGTGGCTCTT-GGAG and CTAAGTTCTT TAGCACTCCTTGGCA; IL-8, CGATGCTGAGCTGATC and CCATGGTTTC-ACCAAAATGATC; mast/stem cell factor receptor, ACA-GAAGCCCTGGTAGACC and GAGGCTGGAGGAG-GAAG; B4-2, AAACCCCCCTCAGGAAAGAC and CCT-ATGAAACAGCTGGCAT; and actin, AGTACTCCGTG-GCATGCC and GCTGATCCACATCTGCTGGA
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21. L.M.A.G.E. clones were amplified by PCR in 96-well format with amino-linked primers at the 5' end. Purified PCR products were suspended at a concentration of ~0.5 mg/ml in 3× SSC, and ~5 ng of each product was arrayed onto coated glass by means of procedures similar to those described previously (22). A total of 9996 elements were arrayed onto an area of 1.8 cm by 1.8 cm with the elements spaced 175 µm apart. The microarrays were then postprocessed to fix the DNA to the glass surface before hybridization with a procedure similar to previously described methods (22).
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